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Metabolic and physiological analyses reveal that *Populus cathayana* males adopt an energy saving strategy to cope with phosphorus deficiency

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Running title: Foliar and root metabolic profiles in poplars

Abstract Dioecious trees have evolved sex-specific adaptation strategies to cope with inorganic phosphorus (Pi) limitation. Yet, little is known about the effects of Pi limitation on plant metabolism, particularly in dioecious woody plants. To identify potential gender-specific metabolites appearing in response to Pi limitation in poplars, we studied the metabolic and ionomic responses in the roots and leaves of *Populus cathayana* males and females exposed to a 60-day period of Pi deficiency. Besides significant decreases in phosphorus contents in both Pi-deficient roots and leaves, the calcium level decreased significantly and the sulfur content increased significantly in Pi-deficient male roots, while the zinc and ferrum contents increased significantly in Pi-deficient female roots. Pi deficiency caused a smaller change in the abscisic acid content, but a significant increase in the jasmonic acid content was detected in both leaves and roots. Salicylic acid significantly decreased under Pi deficiency in male leaves and female roots. Changes were found in phospholipids and phosphorylated metabolites (e.g. fructose-6-phosphate, glycerol-3-phosphate, glucose-6-phosphate, phosphoric acid and inositol-1-phosphate) in roots and leaves. Both *P. cathayana* males and females relied on PPi-dependent but not on Pi-dependent glycolysis under Pi-deficient conditions. Sex-specific metabolites in leaves were primarily in the category of primary metabolites (e.g. amino acids), while in roots primarily in the category of secondary metabolites (e.g. organic acids) and sugars. The metabolome analysis revealed that sexually different pathways occurred mainly in amino acid metabolism, and the tissue-related differences were in the shikimate pathway and glycolysis. We observed changes in carbon flow, reduced root biomass and increased amino acid contents in *P. cathayana* males but not in females, which indicated that males have adopted an energy-saving strategy to adapt to Pi deficiency. Thus, this study provides new insights into sex-specific metabolic responses to Pi deficiency.

Keywords: dioecy, sex-specific responses, metabolome, phosphorus limitation, adaption strategy

Introduction

As one of essential macronutrients, phosphorus (P) plays a central role in almost all aspects of plant physiological and metabolic processes (Netzer et al. 2017). Generally, plants absorb inorganic forms of P (HPO_4^{2-} or $H_2PO_4^-$). However, the supply of inorganic P (Pi) is often not sufficient to meet the requirements for tree growth and development in many terrestrial ecosystems (Batjes 1997, Warren 2011, Prietzel et al. 2016). To cope with the Pi limitation, plants have evolved several metabolic and biochemical strategies to optimize Pi utilization and recycling (Schachtman et al. 1998). For example, the synthesis of sulfolipids can be increased relative to that of phospholipids, changes can occur in the production and exudation of amino acids and organic acids, or alternative types of carbon metabolism can be activated (e.g. sucrose synthase, malate dehydrogenase and glucose-6-phosphate dehydrogenase) (Hernández et al. 2007, Péret et al. 2011, Pant et al. 2015, Esfahani et al. 2016, Ziegler et al. 2016). In addition to the regulation of the activity and abundance of enzymes participating in metabolite production, poplars can also change the expression of specific mRNAs, proteins and posttranscriptional modifications under Pi-deficient conditions (Wei et al. 2013, Chen et al. 2016, Zhang et al. 2016). The expression of genes encoded as Pi transporters (e.g. PHT1;9, PHO1;H1 and PHO2) was induced in the leaves or roots of *Populus simonii* and *Populus* × *euramericana* by Pi deprivation (Gan et al. 2016). Changes in metabolite abundance directly reflects in the plant physiological processes and regulation of gene expression. The generation of flavonoids and condensed tannins can be increased by Pi deficiency in *Populus tremuloides* (Randriamanana et al. 2014). However, there is little information available regarding sexually differential changes in the metabolic profiles of poplars responding to Pi deficiency.

Populus cathayana Rehd. is an ecologically and economically important dioecious species, commonly cultivated in valley or mountain areas of China, where soil P availability is low. *P. cathayana* males and females have differences in stress tolerance and resource demands and they show different physiological and morphological responses to nutrient limitation, male poplars being more tolerant than females (Zhang et al. 2014, Han et al. 2018, Song et al. 2018). Compared with female leaves of *P. cathayana*, male leaves exhibit higher activities of acid phosphatase, nitrate reductase (NiR) and glutamine synthetase (GOGAT) but a lower leaf N:P ratio and less damage to the photosynthetic system under Pi-deficient conditions (Zhang et al. 2014). On the other hand, Pi-deficient males have a higher photosynthetic rate and a better antioxidant and osmotic regulation capacity than Pi-deficient females (Zhang et al. 2014). Our previous proteomic data has indicated that the leaves of *P. cathayana* females exhibit a wider range of protein variation when compared to male leaves under Pi-deficient conditions. These proteins are involved in energy and lipid metabolism, stress responses and gene expression regulation (Zhang et al. 2016). Although the previous studies provided insights into different physiological and molecular processes of poplars in response to Pi deficiency, the exact metabolite profiles remain unknown, particularly for roots.

Roots are more sensitive to signals of nutrient limitation than leaves, and more lateral roots can develop with the arrest of a primary root tip (Abel 2011, Péret et al. 2011, Müller et al. 2015). On one hand, Pi can be transported from old leaves to young leaves at an early stage of deficiency. On the other hand, plants can conserve and scavenge internal Pi through altering their metabolic processes at later stages. It has been reported that phospholipids in biomembranes could be substituted for sulfolipids and galactolipids, and inorganic pyrophosphate (PPi) instead of Pi could be used in metabolic bypass reactions (Plaxton and Tran 2011). However, tissue or gender variability can induce changes in the expression of a set

of genes and associated metabolic processes (Gan et al. 2016, Kavka et al. 2016, Zhang et al. 2016). For example, Pi deficiency increases gene expressions of *PHT2;1*, *AMT2;1* and *NR* in poplar roots, and *PHT1;9*, *AMT1;1* and *NRT2;1* in leaves (Gan et al. 2016, Kavka et al. 2016). Pi-deficient female poplars have been found to show higher abundances of proteins that are involved in lipid metabolism when compared to Pi-deficient males (Zhang et al. 2016). Gene expression is closely related to metabolic change. Therefore, also metabolic profiles may be different between roots and leaves and sexes under Pi deficiency.

Plant nutrient contents are usually assumed to be tightly regulated by the uptake and distribution of nutrients to ensure a relative compositional homeostasis (Maillard et al. 2016). In response to a given nutrient deficiency, up-regulation of non-specific transporters to improve the uptake of a limited nutrient may indirectly increase the uptake of other nutrients (Vrede et al. 2004, Puig et al. 2007, Gojon et al. 2009). It has been reported that P is antagonistic with S, and cooperative with copper (Cu), zinc (Zn), manganese (Mn) and ferrum (Fe) in Pi-deficient tea leaves (Ding et al. 2017). N, K, or S uptake decrease under P-deficiency in the leaves of *B. napus* (Maillard et al. 2016). On the other hand, the cross-talk within the ionomic composition caused by nutrient limitation varies among species and tissues. For example, Pi deficiency increases the root N concentration while it decreases the foliar N concentration (Gan et al. 2016). However, sex-related ionic changes have not received much attention.

According to our previous studies, poplar males have a higher content of substances for osmotic adjustment (e.g. proline, total amino acids and sugars) and a greater expression of stress responsive genes (e.g. HSPs, peroxidase and thaumatin-like protein) than females under stress conditions (Chen et al. 2010, Jiang et al. 2015, Zhang et al. 2014, 2016). We hypothesize now that *P. cathayana* males might have a wider variety of metabolic changes than females in roots

and/or leaves, and these sex-related metabolites may play important roles when coping with Pi deficiency. Therefore, to gain a better understanding and characterization of gender-specific metabolites in *P. cathayana* roots and leaves in response to Pi deficiency, we investigate here the metabolome based on gas chromatography-mass spectrometry (GC-MS) and the ionome based on inductively coupled plasma-optical emission spectrometry (ICP-OES). We will answer the following questions: (1) What are the sex-specific metabolic profiles in roots and leaves? (2) Are changes in sexually differential metabolites related to adaption to Pi deficiency?

Materials and methods

Plant materials and experimental treatments

A completely randomized experimental design with two factors (sex and phosphorus) and two levels (control and deficiency) was established. There were a total of four treatments: control females (FC), control males (MC), Pi-deficient females (FP) and Pi-deficient males (MP). Male and female cuttings of *P. cathayana* were collected from 20 hybridized F_1 individuals (10 males and 10 females). The mother trees were collected from the Qinghai Province, China (LeDu, 36° 31′ N, 102° 28′ E). They were cultured in modified Hoagland solution: 0.25 mM $KH_2(PO_4)$, 1.25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1.25 mM KNO_3, 4.6 μM $MnCl_2{\cdot}4H_2O$, 0.19 μM $ZnSO_4{\cdot}7H_2O$, 0.5 mM $MgSO_4{\cdot}7H_2O$, 0.08 μM $CuSO_4{\cdot}5H_2O$, 11.6 μM H_3BO_3, 10 μM Fe (Fe(III)-EDTA) and 0.12 μM $Na_2MoO_4{\cdot}2H_2O$. The pH was adjusted to 6.5 ± 0.2 using a low concentration of HCl or NaOH (Fodor et al. 2005). After cuttings had grown in the Hoagland solution for 60 days, $KH_2(PO_4)$ was replaced by an equal molar concentration of KCl for Pi-deficient treatments. Each treatment included at least 20 cuttings. The hydroponic solution was changed every three days. The growth conditions were as follows: a daytime temperature of 19-28 °C, a nighttime

temperature of 12-18 °C and a relative humidity of 40-85% in a greenhouse under natural light. According to our previous experiments, sexual differences in morphology were visible after 60 days under Pi deficiency (Fig. S1). To be consistent with our published proteomic data (Zhang et al. 2016), the length of the Pi deficiency treatment lasted 60 days also in this study. The roots, stems and leaves were collected and weighted for biomass measurements. The dried roots and leaves were used for the ionic analysis. The 3-5 rd fully expanded leaves (counted from the top) and root tips (a 5-cm piece) were collected and stored in liquid nitrogen for biochemical and metabolic analyses.

Measurements of biomass, foliar proteins, total amino acids and lipid peroxidation

At the end of the treatments, roots, stems and leaves were harvested and collected into big envelopes. Tissues were dried at 105 °C for 20 min, and then dried to constant weight at 80 °C for biomass measurements. The sum of leaves and stems was considered as aboveground biomass and was used to calculate the aboveground biomass to roots ratio. The soluble protein concentration was determined using the method by Bradford (1976). Fresh leaves (0.2 g) were homogenized in pre-cooled 50 mM phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 100 μM phenylmethanesulfonyl fluoride and 2% polyvinyl pyrrolidone (w/v). The extract was centrifuged at 8000 g at 4 °C for 10 min. The supernatant was collected for the soluble protein measurement. The absorbance was determined at 595 nm using a spectrometer (Unicam UV-330, Unicam, Cambridge, UK). Bovine serum albumin was used as a standard.

The total foliar amino acid content was measured according to Lei *et al*. (2006). Approximately 0.5 g frozen leaves were homogenized in 5 mL of 10% (v/v) acetic acid at 4 °C. After centrifugation (10 000g), 1 mL supernatant was added into 3 mL ninhydrin reaction solution

including 0.1 mL of 0.1% ascorbic acid. The mixture was boiled in a water bath for 15 min and cooled to 25 °C. After 3 mL of 50% ethanol was added, the absorbance was determined at 570 nm. The amount of free amino acids was determined according to a standard curve of arginine.

Lipid peroxidation was determined as the content of thiobarbituric acid reactive substances (TBARS) and measured according to Zhang et al. (2012). Frozen leaves (0.3 g) were homogenized in 4 mL of 20% (v/v) ethanol at 4 °C. After centrifugation (10 000g), 2 mL supernatant was added into the reaction solution, including 20% (w/v) TCA, 0.6% thiobarbituric acid and 0.01% butylated hydroxytoluene. Then, the mixture was boiled for 15 min and quickly cooled to 25 °C. The absorbances were determined at 450, 532 and 600 nm using a spectrometer. The TBARS content was calculated using the following formula: $C = 6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$.

Measurements of mineral nutrients in roots and leaves

To determine the contents of mineral nutrients, poplar roots and leaves were dried using a muffle furnace, and 0.2 g of each tissue sample was subjected to thermal decomposition at 500 °C for 6 h. Then, 10 mL of HNO_3: H_2O_2 (1: 1) was added to each sample, followed by 10-min incubation to extract ions. According to the operation manual, the contents of calcium (Ca), potassium (K), Zn, Fe, P and S were determined using an Agilent 710 ICP-OES spectrometer (Agilent, California, Palo Alto, USA).

Measurements of phytohormones in roots and leaves

The frozen tissues (0.5 g) were soaked in 2 mL pre-cooled extraction solution (containing 0.5%

formic acid and 80% methanol) and ultrasonically extracted for 30 min using 5-mL centrifuge tubes. After centrifugation at 12000 r min^{-1} for 15 min (4 ºC), 2 mL pre-cooled extraction solution was added into the sediment and ultrasonicated again. The supernatant was mixed together, and the aqueous phase was evaporated under 38 ºC using a rotary evaporator (RE-2010, Yuanjian Instrument, Xi'an, China). After freezing for 30 min at -20 ºC, the extraction was thawed and centrifugated at 12000 r min^{-1} for 10 min (4 ºC). The supernatant was concentrated to near dry using a termovap sample concentrator (DC150-1A, Yooning Instrument, Hangzhou, China), then 1 mL acetonitrile was added and the sample was ultrasonicated for 30 s. Finally, the extraction was filtered through a 0.22 μm millipore filter. The analysis of hormones was performed by Agilent 1260LC with Agilent ZORBAX Eclipse C18 (150 × 4.6 mm, 5 μm) (Agilent Technologies, Santa Clara, CA, USA). Two mobile phases consisting of 0.1% formic acid (A) and acetonitrile (B) were used for gradient elution at a flow rate of 0.5 ml min^{-1}. The contents of abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) were determined relative to the corresponding internal standard.

Metabolite extraction from roots and leaves

Metabolites were extracted according to Lisec et al. (2006) with some modifications. After 0.1 g of frozen leaves or roots were homogenized in a mortar with liquid nitrogen, they were transferred into 10-ml centrifuge tubes. Metabolites were extracted in 1400 μL of 100% pre-cooled methanol and vortexed for 30 s. Subsequently, 60 μL of ribitol (0.2 mg ml^{-1}) was added as the internal quantitative standard. After the mixture was ultrasonically treated for 15 min, 750 μL of chloroform (-20 °C) and 1500 μL of dH_2O (4 °C) were added, followed by centrifugation for 15 min at 4000 g. The supernatant was transferred into 2-mL tubes. After drying in a termovap sample concentrator (DC150-1A, Yooning Instrument, Hangzhou, China),

60 μL of 15 mg mL^{-1} methoxyamine hydrochloridein pyridine was added, followed by incubation at 37 °C for 16 h. Finally, 60 μL of BSTFA (including 1% chlorotrimethylsilane) was added into the mixture, which was kept for 60 min at room temperature.

GC-MS and metabolic profile analysis

Metabolites were determined using an Agilent 7890A GC/5975C MS system (Agilent, California, Palo Alto, USA). The extract (1 μL) was injected into a HP-5 MS capillary column (5% phenyl methyl silox). The split ratio was 20:1. The column was 30 m × 0.25 mm ID and 0.25 μm df with 10-m Integra-Guard (Agilent J & W scientific, Folsom, CA). The injection temperature was set to 280 °C, the MS source to 250 °C, and the MS quad to 150 °C. The oven temperature program was as follows: initial isothermal heating at 80 °C for 5 min, followed by a 20 °C min^{-1} ramp to 300 °C and heating at 300 °C for 6 min. The total running time was 22 min. Helium (1.0 ml min^{-1}) was used as the carrier gas. Mass spectrometry was conducted using a full-scanning method with a range from 35 to 780 (m/z). The database of NIST (http://www.nist.gov/index.html) and KEGG (http://www. genome.jp/kegg/) were used for searching and identifying metabolites.

The raw signal extraction, data filtering and peak identification were conducted using the XCMS software (www.bioconductor.org/). Except for the default parameters of the XCMS software, some modifications were used as follows: xcmsSet (fwhm = 3, snthresh = 3, mzdiff = 0.5, step = 0.1, steps = 2, max = 300), retcor (method = obiwarp, plottype = c (deviation), bandwidth (bw) = 2, minfrac = 0.3). For multiple linear regression analyses, the final data were imported into Excel tables. To ensure the accuracy and consistency of the identified true chemical entities and to remove misassignments and background noise, a QC was designed.

With the integral area of Ribitol normalized to 1000, each sample was normalized by the total mass of the signal integration area. The normalized data were imported into Simca-P 11.0 software (Umetrics AB, Umea, Sweden), and ANOVA analyses were used to identify the effects of sex, Pi-deficiency and their interaction in leaves and roots.

Statistical data analyses

For biomass, biochemical, phytohormone and metabolite measurements, five biological replicates were used. For ion measurements, four biological replicates were used. The significance of differences in the measured parameters between treatments and controls were tested using ANOVA with Tukey's test at $p < 0.05$ level by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). All metabolic data were standardized using the Simca-P software (version 11.0, http://www.umetrics.com/simca). The principal component analysis (PCA) was performed to display variation patterns in the metabolite data. The partial least squares discrimination analysis (PLS-DA) was conducted to determine compounds that separated the control and treated roots and leaves, respectively. Quantitative normalization within replicates was achieved, and the software of MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/faces/ModuleView.xhtml) was used to build heat map diagrams and metabolite profiles (Chong et al. 2018).

Results

Morphological and biochemical changes in responses to Pi deficiency

After a 60-day period of Pi deficiency in a hydroponic solution, leaves of both sexes were

thickened but they remained green, but the petiole became red in *P. cathayana* females (Fig. S1). Compared with controls and males, the total biomass of Pi-deficient females decreased more, which was mainly due to a decline of stem biomass (Fig. S2). Although fewer lateral roots and root hairs were produced under Pi deficiency, root biomass increased in both sexes, leading to a decrease in aboveground mass to root ratio (Fig. 1A). Additionally, Pi deficiency caused an increase in the foliar total protein content of males, while no significant increase was found in females (Fig. 1B). The total free amino acid content decreased significantly, while TBARS increased significantly in Pi-deficient female leaves (Fig. 1C and D), indicating that *P. cathayana* females suffered from greater negative effects under Pi limitation compared to males.

Mineral nutrient changes in responses to Pi deficiency

Under control conditions, all ion contents showed minor sexual differences in leaves (Fig. 2). However, the Ca and Zn contents were significantly higher and P and K were significantly lower in male roots when compared to female roots (Fig. 3). Pi deficiency greatly changed ionic equilibrium in both leaves and roots. In leaves, the P content decreased significantly in males, while Zn and Fe contents increased significantly in females under Pi-deficient conditions (Fig. 2). Interestingly, Pi-deficient female leaves exhibited a higher S content than males, although there was a smaller sexual difference under control conditions. In roots, Pi deficiency significantly decreased the P content in both sexes and Ca content in males, and it increased the S content of females but not significantly (Fig. 3). Pi-deficient female roots had a higher K content but lower Zn and Fe contents compared to Pi-deficient male roots.

Phytohormone changes in responses to Pi deficiency

300 Under control conditions, there were minor sexual differences in the contents of ABA, JA and
301 SA in leaves, while male roots showed higher ABA and lower SA contents than female roots
302 (Fig. 4). Pi deficiency caused only a small change in ABA but a significant increase in JA in
303 both leaves and roots. SA decreased significantly under Pi deficiency in male leaves and female
304 roots. Compared to females, Pi-deficient males had a higher JA content in leaves but a lower
305 content in roots.
306
307 *Metabolic changes in responses to Pi deficiency*
308
309 In our study, 231 compounds (peaks) were detected in either roots or leaves of *P. cathayana* by
310 GC-MS, and 90 metabolites were successfully identified (Table S1). To illustrate visually sex-
311 and tissue-related differences, data on all metabolites (consisting of 20 samples) were used for
312 PCA and a hierarchical cluster analysis. The results of PCA showed that samples could be
313 clearly separated by PC1, which explained approximately 56.3% of the variability (Fig. S3).
314 Heatmap showed that the most obvious separation was the highest-order clustering according
315 to tissue, followed by sex and finally by treatment (Fig. 5). Both PCA and the horizontal cluster
316 showed a clear separation between roots and leaves, and then between sexes. To better show
317 the presence of sex-related metabolites under Pi deficiency, PLS-DA was then performed for
318 roots and leaves (Fig. 6). In leaves, the contribution of metabolites to PC1 was 28.60%, coming
319 primarily from amino acids and phosphate compounds, and to PC2 13.80%, coming primarily
320 from sugars. In roots, the contribution of metabolites to PC1 was 28.60%, coming primarily
321 from organic acids and phosphate compounds, and PC2 (18.20%) came primarily from amino
322 acids.
323
324 *Sexually different metabolites in leaves*

According to the ANOVA analysis, the significances of metabolites affected by sex, Pi deficiency and their interaction are listed in Table S1. Under control conditions, 9 amino acids, 6 organic acids, 2 polyols (digalactosylglycerol and galactosylglycerol), ethanolamine, glycerol-3-phosphate (G3P) and isomaltose had significantly higher abundances in female leaves than in males leaves (Table 1 and Fig. 7). Three organic acids (alpha-ketoglutaric acid, benzoic acid and glucaric acid), triethanolamine, glucose-6-phosphate (G6P), phosphoric acid (H_3PO_4), iaminaribiose and melezitose had significantly lower contents in female leaves than in male leaves. Under Pi-deficient conditions, 13 and 7 metabolites showed significant down-regulation in female and male roots, respectively. Four metabolites showed significant up-regulation in male roots but none in female roots. Among these, the common down-regulated metabolites caused by Pi-deficiency in both sexes were G6P, H_3PO_4, digalactosylglycerol and glycerol. Additionally, most amino acids showed significantly higher contents in female leaves than in male leaves under control conditions, but there was no statistical difference under Pi-deficient conditions. Interestingly, 5 metabolites (4-hydroxy-3-methoxybenzoic acid, 4-hydroxybenzoic acid, threonic acid, digalactosylglycerol and galactosylglycerol) had significantly higher contents, while 4 metabolites (alpha-ketoglutaric acid, benzoic acid, glucaric acid and melezitose) possessed significantly lower contents in female leaves than in male leaves under both control and Pi-deficient conditions.

Sexually different metabolites in roots

Under control conditions, 15 and 18 metabolites showed significantly higher and lower abundances in female roots than in male roots, respectively (Table 1 and Fig. 8). Compared with male roots, the highly abundant metabolites in female roots were primarily involved in

phosphate compounds and sugars, and the low-abundance metabolites were primarily involved in organic acids. Under Pi-deficient conditions, 16 and 12 metabolites were significantly down-regulated, and 4 and 8 metabolites were significantly up-regulated in female and male roots, respectively. Among these, valine, ethanolamine, inositol-1-phosphate, H_3PO_4, glycerol and arabinose were commonly down-regulated, while 4-hydroxy-3-methoxybenzoic acid and sucrose were commonly up-regulated in both Pi-deficient male and female roots. The most increased metabolites caused by Pi-deficiency were dulcitol (42.74 folds) and sucrose (20.20 folds) in male and female roots, respectively. The most decreased metabolites were G6P (141.54 folds), G3P (43.73 folds), fructose-6-phosphate (37.83 folds) and inositol-1-phosphate (23.83 folds) in female roots, and inositol-1-phosphate (9.10 folds) and H_3PO_4 (9.01 folds) in male roots. Particularly, 4 metabolites (ethanolamine, monomethylphosphate, fructose and glucose) showed higher contents and 7 metabolites (urea, pyruvic acid, threonic acid, arabinitol, xylitol, iaminaribiose and rhamnose) showed lower contents in female roots than in male roots under both control and Pi-deficient conditions.

Discussion

Sexually different changes in mineral nutrients and phytohormones under Pi deficiency

Plant nutrient contents are usually assumed to be tightly regulated by the uptake and distribution of nutrients to ensure a relative compositional homeostasis (Maillard et al. 2016). In deciduous trees, Pi can be reused and remobilized from the old leaves to young ones when they suffer from nutrient limitation, but such remobilization does not happen for roots (Cherbuy et al. 2001). This may be one of the reasons for the greater P decrease in roots when compared to leaves in both sexes. Under good nutrient conditions, *P. cathayana* males showed higher Ca and Zn

375 contents while lower P and K contents in leaves compared to females, indicating sexually
376 differential needs for mineral nutrition to meet the requirements for growth. The energy
377 provided by Ca^{2+}-ATPase is necessary for the absorption and transport of Ca^{2+}, while Pi
378 deficiency decreases Ca^{2+}-ATPase activity in plant cells (Zeng et al. 2013), thus leading to a
379 decrease in the Ca content, as shown in Fig. 3. The S content increased significantly in female
380 roots under Pi-deficient conditions. Sulfur is a component of many important biological
381 compounds, including amino acids and the tripeptide glutathione (Amtmann and Armengaud
382 2009). Such disorder of nutrient equilibrium will further lead to a metabolic disturbance.
383
384 SA and JA are primary phenolics with important defensive functions against biotic and abiotic
385 stresses in the leaves of the Salicaceae family (Boeckler et al. 2011). It has been reported that
386 N deficiency could reduce the SA content in *P. tremula* (Randriamanana et al. 2014). Our data
387 indicated that Pi deficiency caused a greater decrease of SA in female roots than in male roots.
388 However, the roles of SA and JA are often antagonistic in plant resistance (Pieterse et al. 2012).
389 For example, rust disease causes an increase in SA but no change in JA in the leaves of black
390 poplar (Eberl et al. 2017). In our study on *P. cathayana*, we found that SA decreased while JA
391 increased in both roots and leaves, suggesting an antagonistic change between these two types
392 of phytohormones. Interestingly, JA increased most in male leaves and female roots under Pi
393 deficiency, indicating a sex-specific response pattern (Fig. 4). Additionally, Pi deficiency has
394 been proposed to induce the JA pathway and trigger resistance to insect herbivory in
395 *Arabidopsis thaliana* (Khan et al. 2016). Therefore, it is worth being explored how sex- and
396 tissue-specific JA changes caused by Pi deficiency may enhance tolerance against biotic or
397 abiotic stresses in poplars. Metabolically, the sexually different SA and JA responses can be
398 attributed to varying benzoic acid and phytodienoic acid contents in males and females, as these
399 two organic acids are intermediates of the synthesis of SA and JA (Vlot et al. 2009, Ullah et al.

2019).

Common metabolic changes in males and females under Pi deficiency

Under Pi-deficient conditions, phospholipids can be replaced by sulfolipids or galactolipids in biomembranes, which bypass ATP-consuming steps and use inorganic pyrophosphate (PPi) instead of Pi (Plaxton and Podestá 2006, Plaxton and Tran 2011). In this study, we found reductions in several metabolites involved in membrane phospholipid metabolism in both roots and leaves of *P. cathayana* under Pi-deficiency, e.g., arabinose, inositol-1-phosphate and ethanolamine. This energy saving pathway can maintain physiological activities even under low ATP and Pi levels (Florez-Sarasa et al. 2014, Wang et al. 2014). With this strategy, plants can maintain the carbon flow down to the citric acid cycle, which is proved by an increased amount of citric acid in roots.

Gycerol-3-phosphate (G3P), F6P and G6P, involved in glycolysis and in the biosynthesis of phospholipids of biomembranes, were significantly reduced in the leaves and roots of both sexes under Pi-deficient conditions (Table 1 and 2). As mentioned above, plant glycolysis can use a PPi-dependent but not a Pi-dependent process under Pi-deficiency. Sucrose synthase and phosphoenolpyruvate carboxylase, involved in glycolytic bypass reactions, were induced to higher levels under Pi-deficiency (Zhang et al. 2016), suggesting that glycolysis is still maintained in Pi-deficient leaves. Possibly, F6P, G3P and G6P are metabolized rapidly by PPi-dependent glycolysis under Pi-deficiency, which would explain the great decrease in their abundance in both sexes. Additionally, as a crucial component of glycerophospholipids of biomembranes, G3P plays an important role in plant cells. It has been reported that the phospholipids of biomembranes could be remodeled and replaced by galactolipid

digalactosyldiacylglycerol to maintain membrane integrity under Pi-deficient conditions (Nakamura et al. 2009, Lan et al. 2012). Notably, we found that digalactosylglycerol significantly increased in roots (Table 2), which supports the view of membrane remodeling induced by Pi deficiency, although the process of remodeling is not clear.

Sexually different metabolic responses to Pi-deficiency

The defense theory predicts that to maximize fitness, plants distribute their defensive substances to the organs at the highest risk for predation and to those with the highest fitness value (Stamp 2003). Aconitic acid and caffeoylquinic acid, two newly reported defense-responsive organic acids against biotic and abiotic stresses (Li et al. 2017, Zhang et al. 2018), were significantly up-regulated in male leaves but remained unchanged in female leaves under Pi-deficient conditions (Fig. 7), indicating that male poplars would be more tolerant to stresses and would have a higher fitness value than females.

The most sex-specific metabolites in leaves were in the category of amino acids (Fig. 7). The identified amino acids (except for tyrosine) were down-regulated in Pi-deficient female leaves but up-regulated in male leaves. Our previous study has indicated that *P. cathayana* males possess relatively higher photosynthetic rates and photorespiration than females under Pi-deficient conditions (Zhang et al. 2014). The increase in photorespiratory flux can promote amino acid synthesis, particularly that of glutamic acid, and ammonia re-assimilation (Tcherkez et al. 2008). Therefore, the increase of soluble amino acids in male leaves could be due to increased N assimilation. On the other hand, the increase in putrescine and free amino acids is proposed to result from protein degradation and would eliminate ammonia toxicity in Pi-deficient male leaves (Alexova et al. 2017). Our previous proteomic data provided evidence

that several chaperonin proteins involved in protein degradation were up-regulated in Pi-deficient male leaves but down-regulated in Pi-deficient female leaves (Zhang et al. 2016). For example, chaperonin 60, chaperone protein ClpC1 and T-complex protein 1 were greatly up-regulated in Pi-deficient male leaves, whereas kunitz-type protease inhibitor was greatly up-regulated in Pi-deficient female leaves. This outcome may indicate that *P. cathayana* males use a protein degradation strategy to cope with Pi deficiency in leaves.

The success of Pi-limited plants depends on their ability to maintain photosynthetic rate and transport carbohydrates to growing roots (Alexova et al. 2017). Under Pi-deficient conditions, compared with females, *P. cathayana* males possess a higher photosynthetic rate but less root biomass (Zhang et al. 2014). The assimilated photosynthetic carbohydrates might be diverged to secondary metabolite synthesis rather than to be used for the root growth of males. The carbohydrate export to roots is an energetically costly process, as approximately 20-40% of foliar ATP is used for membrane transport (Noguchi et al. 2001). Long-lasting Pi deficiency has been shown to reduce protein anabolism but to enhance the production of secondary metabolites (Müller et al. 2015). Thus, the increase in protein degradation in Pi-deficient *P. cathayana* males could represent a significant energy-saving strategy and offset the energy investment to roots.

The most sex-specific metabolites of roots were in the shikimate pathway and glycolysis (Fig. 8). For example, benzoic acid was up-regulated in female roots, while maleic acid was down-regulated in male roots. It has been proposed that the accumulation of polyols and sugars in plant tissues can efficiently regulate osmotic pressure caused by stress (Holmstrup et al. 2001). The significant increases in lactic acid, digalactosylglycerol, dulcitol and xylitol in male roots but not in female roots indicate that males have a better osmotic regulation under Pi-deficient conditions.

It has been widely reported that an increase in organic acid exudation into the rhizosphere can promote Pi solubilization in soil (Playsted et al. 2006, Meyer et al. 2010, Oburger 2011). Our data showed that the contents of several organic acids involved in the tricarboxylic acid (TCA) cycle and shikimate pathway increased significantly in roots (Fig. 8). Interestingly, 4-hydroxy-3-methoxybenzoic acid increased in roots but decreased in leaves, which is consistent with previous reports that poplar cuttings possess different phenolic profiles in roots, stems and leaves in response to Pi limitation (Tsai et al. 2006, Randriamanana et al. 2014). According to the protein competition hypothesis, growth proteins and defense phenolics compete for the common precursors, particularly in nutrient-deficient conditions (Jones and Hartley 1999, Wright et al. 2010). Because of a constant competition for C skeletons between N assimilation (e.g. amino acid pool), TCA cycle (e.g. organic acid pool) and phosphoenolpyruvate (Le Roux et al. 2006, 2008, Valentine et al. 2017), an increase in organic acids would take place at the expense of N fixation. This hypothesis well explains the increase in organic acids and the decrease in amino acids in poplar roots (Fig. 8). Additionally, Pi limitation leading to restrained N assimilation possibly contributes to a low expression or abundance of NiR and GOGAT (Wu et al. 2003, Zhang et al. 2014, Gan et al. 2016). Therefore, carbohydrates are favored to flow toward organic acid synthesis rather than N-metabolism in Pi-stressed poplar roots.

Conclusions

We found sex-specific changes in the ionome and metabolome of *P. cathayana* leaves and roots in response to Pi deficiency. The commonly changed metabolites were phospholipids in both roots and leaves. Pi-deficient males and females rely on PPi-dependent rather than Pi-dependent glycolysis. The sex-specific metabolites in leaves were primarily in the category of primary

metabolites (amino acids), and in roots secondary metabolites (organic acids) and sugars. These sexually differential changes in metabolites provide new evidences for the better tolerance of male poplars to Pi deficiency when compared to females. Therefore, considering the global environmental change and P limitation in soil, there is a reason to predict that sex-specific growth and trade-off patterns will be enhanced among dioecious poplars, especially among young trees.

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Conflict of interest None declared.

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750 **Table 1**. Fold changes of DEMs in leaves of *P. cathayana* males and females under control and

751 Pi-deficient conditions (n=5).

Metabolites/Category	Fold changes [1]				ANOVA *p* values [2]		
	FC/MC	FP/MP	FP/FC	MP/MC	p_s	p_p	$p_{s\times p}$
Amino acids							
Alanine	7.41*	1.17	0.49*	3.07	0.00	0.19	0.00
Glycine	2.43*	1.04	0.50*	1.18	0.00	0.02	0.00
Isoleucine	5.63*	0.96	0.60	3.51	0.00	0.85	0.00
Leucine	3.42*	1.93	0.64	1.12	0.00	0.26	0.18
Proline	8.07*	1.24	0.52*	3.37	0.00	0.36	0.00
Serine	6.65*	0.71	0.44	4.13	0.05	0.78	0.00
Threonine	3.17*	0.59	0.45*	2.39	0.18	0.68	0.00
Tyrosine	2.58*	1.78	0.61*	0.89	0.00	0.04	0.09
Valine	5.15*	1.12	0.49*	2.25	0.00	0.17	0.00
Amines							
Ethanolamine	3.63*	2.52	0.48*	0.69	0.00	0.01	0.06
Putrescine	1.87	0.85	1.09	2.41*	0.28	0.00	0.01
Triethanolamine	0.41*	0.98	1.52	0.63*	0.00	0.40	0.01
Nucleotides							
1-Benzylglucopyranoside	0.42	0.39*	1.28	1.38	0.00	0.19	0.49
Adenosine	0.74	0.38*	0.52	1.00	0.00	0.15	0.14
Organic acids							
2-methyl-butanedioic acid	1.55*	0.94	0.77	1.27	0.02	0.62	0.00
4-Hydroxy-3-methoxybenzoic acid	4.08*	3.15*	0.77*	1.00	0.00	0.03	0.03
4-Hydroxybenzoic acid	6.46*	5.62*	0.83	0.96	0.00	0.37	0.41
Aconitic acid	0.63	0.30*	0.95	2.00*	0.00	0.06	0.04
alpha-ketoglutaric acid	0.24*	0.20*	1.06	1.26	0.00	0.23	0.27
Benzoic acid	0.36*	0.32*	1.03	1.14	0.00	0.27	0.34
Caffeoylquinic acid	0.93	0.55*	1.03	1.74*	0.00	0.01	0.01
Eicosanoic acid	0.74	0.49*	0.79	1.20	0.00	0.79	0.04
Glucaric acid	0.37*	0.36*	1.11	1.13	0.00	0.39	0.64
Gluconic acid	1.58	1.69	0.93	0.87*	0.00	0.45	0.96
Itaconic acid	0.85	0.66*	0.83	1.08	0.01	0.71	0.24
Maleic acid	4.59*	1.26	0.34*	1.26	0.01	0.05	0.03
Threonic acid	2.13*	2.97*	0.84	0.60	0.00	0.15	0.91
Phosphate compounds							
Glucose-6-phosphate	0.69*	0.63	0.39*	0.43*	0.01	0.00	0.31
Glycerol-3-phosphate	4.50*	2.01	0.44*	0.98	0.00	0.03	0.03
Inositol-1-phosphate	1.05	0.68	0.49*	0.76	0.44	0.01	0.25
Phosphoric acid	0.65*	0.71	0.47*	0.44*	0.00	0.00	0.09
Polyols							
1-Monohexadecanoylglycerol	0.84	0.98	1.42*	1.22	0.20	0.00	0.33

2-Methyl-1,3-butanediol	0.72	1.12	0.79	0.51*	0.34	0.01	0.14
Digalactosylglycerol	2.97*	2.59*	0.67*	0.77*	0.00	0.02	0.12
Galactosylglycerol	2.99*	3.26*	0.71*	0.65	0.00	0.01	0.22
Glycerol	1.25	1.36	0.52*	0.48*	0.03	0.00	0.68
Sugars							
Iaminaribiose	0.60*	0.72	1.15	0.96	0.00	0.80	0.48
Isomaltose	2.38*	1.52	0.91	1.43	0.00	0.69	0.25
Melezitose	0.01*	0.02*	2.13	1.41	0.00	0.14	0.17

1. FC/MC, control females compared with control males; FP/MP, Pi-deficient females compared with Pi-deficient males; FP/FC, Pi-deficient females compared with control females; MP/MC, Pi-deficient males compared with control males; *, a statistical difference at $p < 0.05$ level between the mean of the two treatments.
2. Two-way ANOVAs were performed with Tukey's test. p_s, sex effect; p_p, Pi effect; $p_{s\times p}$, sex and Pi interaction effect.

773 **Table 2**. Fold changes of DEMs in roots of *P. cathayana* males and females under control and

774 Pi-deficient conditions (n=5).

Metabolites	Fold changes				ANOVA *p* values		
	FC/MC	FP/MP	FP/FC	MP/MC	p_s	p_p	$p_{s\times p}$
Amino acids							
Alanine	2.14	0.89	0.30*	0.72	0.09	0.01	0.06
Isoleucine	0.72	0.73	0.65	0.64*	0.02	0.00	0.54
Phenylalanine	0.67*	1.44	0.66	0.31*	0.18	0.00	0.00
Valine	0.70*	0.69	0.56*	0.57*	0.00	0.00	0.30
Amines							
Ethanolamine	1.49*	2.23*	0.62*	0.41*	0.00	0.00	0.92
Urea	0.15*	0.47*	3.14	0.98	0.00	0.15	0.10
Nucleotides							
3-hydroxy-pyridine	1.73*	1.47	0.72	0.85	0.00	0.04	0.25
Uracil	0.62*	0.84	0.75	0.55*	0.01	0.00	0.10
Organic acids							
4-Aminobutyric acid	2.54*	1.65	0.24*	0.37	0.01	0.00	0.06
4-Hydroxy-3-methoxybenzoic acid	1.39	1.11	2.17*	2.71*	0.18	0.00	0.87
Benzoic acid	0.42*	0.70	2.09*	1.25	0.00	0.00	0.34
Caffeic acid	0.63*	1.07	1.15	0.68	0.09	0.22	0.03
Citric acid	3.33	1.95	1.55*	2.64	0.01	0.03	0.91
Galactaric acid	0.51*	0.35	0.33	0.48*	0.00	0.00	0.38
Gluconic acid	0.52*	0.35	0.33	0.49*	0.00	0.00	0.40
Glyceric acid	1.29	0.72	0.47*	0.85	0.79	0.00	0.01
Lactic acid	1.11	0.62	1.07	1.90*	0.20	0.04	0.08
Maleic acid	0.26*	0.75	1.28	0.45*	0.00	0.03	0.01
Malic acid	1.10	0.87	1.45	1.84*	0.71	0.00	0.38
Malonic acid	0.71*	0.90	0.99	0.78	0.01	0.09	0.10
Oxalic acid	1.33	1.02	0.49*	0.64	0.21	0.00	0.24
Pyroglutamic acid	1.84	2.25*	1.19	0.97	0.00	0.54	0.47
Pyruvic acid	0.51*	0.55*	0.96	0.87	0.00	0.32	0.48
Ribonic acid	3.66*	0.81	0.24*	1.09	0.00	0.00	0.00
Shikimic acid	1.06	0.78	1.44	1.96*	0.40	0.01	0.28
Threonic acid	0.25*	0.15	0.62	1.07	0.00	0.87	0.36
Phosphate compounds							
Fructose-6-phosphate	4.05*	0.80	0.03*	0.13	0.00	0.00	0.00
Glucose-6-phosphate	4.54*	0.51	0.01*	0.06	0.00	0.00	0.00
Glycerol-3-phosphate	6.19*	1.06	0.02*	0.13	0.00	0.00	0.00
Inositol-1-phosphate	2.01*	0.77	0.04*	0.11*	0.00	0.00	0.00
Monomethylphosphate	2.56*	5.80*	0.90	0.40	0.00	0.16	0.54
Phosphoric acid	2.47*	1.75	0.08*	0.11*	0.00	0.00	0.00
Polyols							
Arabinitol	0.45*	0.19*	0.33	0.80	0.00	0.01	0.50
Digalactosylglycerol	0.74	0.44*	1.40	2.36*	0.00	0.00	0.00

Dulcitol	1.32	0.07	2.14	42.74*	0.08	0.05	0.07
Glycerol	0.90	0.71	0.47*	0.60*	0.01	0.00	0.38
Maltitol	2.39*	1.15	0.33*	0.69	0.00	0.00	0.00
Xylitol	0.36*	0.23*	1.24	1.92*	0.00	0.00	0.01
Sugars							
Arabinose	1.28	1.14	0.41*	0.46*	0.05	0.00	0.21
Fructose	2.22*	1.73*	0.91	1.17	0.00	0.92	0.30
Galactinol	1.47*	0.98	0.65*	0.98	0.05	0.03	0.04
Glucose	2.41*	1.54*	0.94	1.47	0.00	0.41	0.12
Iaminaribiose	0.52*	0.51*	1.25	1.27	0.00	0.04	0.46
Melezitose	0.49	0.24*	0.65	1.34	0.01	0.74	0.30
Rhamnose	0.53*	0.34*	0.53	0.83	0.00	0.01	0.60
Sucrose	0.59*	1.61*	20.20*	7.34*	0.06	0.00	0.03
Other							
Catechine	4.30*	1.69	0.84	2.15	0.00	0.63	0.08

1. FC/MC, control females compared with control males; FP/MP, Pi-deficient females compared with Pi-deficient males; FP/FC, Pi-deficient females compared with control females; MP/MC, Pi-deficient males compared with control males; *, a statistical difference at $p < 0.05$ level between the mean of the two treatments.
2. Two-way ANOVAs were performed with Tukey's test. p_s, sex effect; p_p, Pi effect; $p_{s\times p}$, sex and Pi interaction effect.

Figure legends

Figure 1. The aboveground mass to root mass ratio (A), total protein content (B), amino acid content (C) and TBARS content (D) in leaves of *P. cathayana* females and males under control and Pi-deficient conditions (n=5). Values denoted by different letters indicate significant differences at $p < 0.05$ according to Tukey's test. P_s, sex effect; P_p, Pi effect; $P_{s\times p}$, sex and Pi interaction effect.

Figure 2. Mineral nutrient contents (μg g^{-1} Dw) in leaves of *P. cathayana* females and males under control and Pi-deficient conditions (n=4). Values denoted by different letters indicate significant differences at $p < 0.05$ according to Tukey's test. P_s, sex effect; P_p, Pi effect; $P_{s\times p}$, sex and Pi interaction effect; Dw, dry weight.

Figure 3. Mineral nutrient contents (μg g^{-1} Dw) in roots of *P. cathayana* females and males under control and Pi-deficient conditions (n=4). Values denoted by different letters indicate significant differences at $p < 0.05$ according to Tukey's test. P_s, sex effect; P_p, Pi effect; $P_{s\times p}$, sex and Pi interaction effect; Dw, dry weight.

Figure 4. Phytohormone contents (ng g^{-1} Fw) in leaves and roots of *P. cathayana* females and males under control and Pi-deficient conditions (n=5). Values denoted by different letters indicate significant differences at $p < 0.05$ according to Tukey's test. P_s, sex effect; P_p, Pi effect; $P_{s\times p}$, sex and Pi interaction effect; Fw, fresh weight.

Figure 5. Heatmap analysis for the detected metabolites in leaves and roots of *P. cathayana* females and males under control and Pi-deficient conditions (LFP, Pi-deficient female leaves,

LFC, control female leaves, LMP, Pi-deficient male leaves, LMC, control male leaves, RFP, Pi-deficient female roots, RFC, control female roots, RMP, Pi-deficient male roots, RMC, control male roots).

Figure 6. PLS-DA score plots of metabolic profiles in leaves (A) and roots (B) of *P. cathayana* females and males under control and Pi-deficient conditions. LMC, control male leaves; LMP, Pi-deficient male leaves; LFC, control female leaves; LFP, Pi-deficient female leaves; RMC, control male roots; RMP, Pi-deficient male roots; RFC, control female roots; RFP, Pi-deficient female roots.

Figure 7. Metabolic pathways in leaves of *P. cathayana* females and males after a 60-day Pi deficiency. The content of each metabolite is its normalized value (n=5). The blue bars indicate control individuals; males are on the left and females on the right. The red bars indicate Pi-deficient individuals; males are on the left and females on the right.

Figure 8. Metabolic pathways in roots of *P. cathayana* females and males after a 60-day Pi deficiency. The content of each metabolite is its normalized value (n=5). The blue bars indicate control individuals; males are on the left and females on the right. The red bars indicate Pi-deficient individuals; males are on the left and females on the right.

Supplementary materials

Figure S1. Morphological traits of *P. cathayana* males and females after a 60-day Pi deficiency. FC, control female; FP, Pi-deficient female; MC, control male; MP, Pi-deficient male.

Figure S2. The biomass of roots, stems and leaves of *P. cathayana* females and males under control and Pi-deficient conditions (n=5). Values denoted by different letters indicate significant differences at $p < 0.05$ according to Tukey's test. P_s, sex effect; P_p, Pi effect; $P_{s\times p}$, sex and Pi interaction effect.

Figure S3. PCA score plots of metabolic profiles in *P. cathayana* females and males under control and Pi-deficient conditions. LMC, control male leaves; LMP, Pi-deficient male leaves; LFC, control female leaves; LFP, Pi-deficient female leaves; RMC, control male roots; RMP, Pi-deficient male roots; RFC, control female roots; RFP, Pi-deficient female roots.

Table S1. Results of foliar and root metabolites identified by GC-MS in *P. cathayana* males and females under control and Pi-deficient conditions. Each metabolite value is the mean and SE (n = 5).